

Simple sequence repeats analysis of two *Paraschistura* sp. populations from Kheirabad and Brim Rivers, Persian Gulf Basin, Iran

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Abstract: The aim of this study was to access the genetic differentiation of *Paraschistura* sp. in Kheirabad and Brim Rivers (Zohreh River System, Persian Gulf Basin), Iran. For this purpose, 60 samples including 30 male and 30 female samples from each river were collected. A total of six microsatellite loci (Bbar4, Bbar7, IC228, IC230, IC434 and IC720) were used and all of them showed high polymorphism (PIC= 0.87). The average numbers of observed alleles per loci in Kheirabad and Brim rivers were 13.5 and 12.5, respectively. The mean of observed heterozygosity values were 0.53 and 0.59 in Kheirabad and Brim rivers, respectively. The bottleneck analysis did not show any evidence of genetic bottleneck in these two rivers. Approximately all of loci showed deviation from Hardy-Weinberg equilibrium (HWE). The genetic distance between the two populations was 0.629, indicating high genetic diversity of these two populations. The results of Molecular Variance Analysis revealed that genetic diversity within populations was 97 percent while between them was 3 percent. The F_{ST} value was 0.02 that indicates the low genetic differentiation between the two populations that could be explained by historical migrations in the past times. Also, the number of migrants (N_m) between the two populations was obtained 9.142. Finally, it seems that *Paraschistura* sp. has an adequate level of genetic diversity in the studied habitats, but the conservation programs are needed to ensure survival of this species in all of its geographical distributions.

Keywords: Genetic diversity, Zohreh River System, Microsatellite, *Paraschistura* sp.

Introduction

Loaches of Iran have been considered in two families including Nemachilidae and Cobitidae. *Paraschistura* is one of six genera belonging to Nemachilidae (Prokofiev 2009; Esmaeili et al. 2010). This genus is recently described and not all species have been examined and ascribed to it or related genera (Coad 2014). *Paraschistura* is characterized in particular by a dark black spot or strip always present at the base of the anterior dorsal fin rays. Members of the genus *Paraschistura* have been recorded from Dasht-e Lut basin and probably exists in most of the Iranian basins (Coad 2014).

Genetic diversity is one of three levels of the biodiversity (Gunderina 2003). This level of biodiversity reveals differences in number and type of alleles presence on chromosome. The management of animal genetic diversity is depended on evaluation of the genetic structure and classification stocks of desired species (Pujolar et al. 2009). Genetic diversity is crucial for evolutionary processes of the environment and the ecology within the ecosystem scale (Rezvani et al. 2012). Recently, microsatellite DNA markers or Simple Sequence Repeats (SSRs) for population genetic studies have high usage due to their unique features such as high polymorphism,

Table 1. Primer sequences, annealing temperatures (°C), PCR product size range of microsatellite loci examined in the present study.

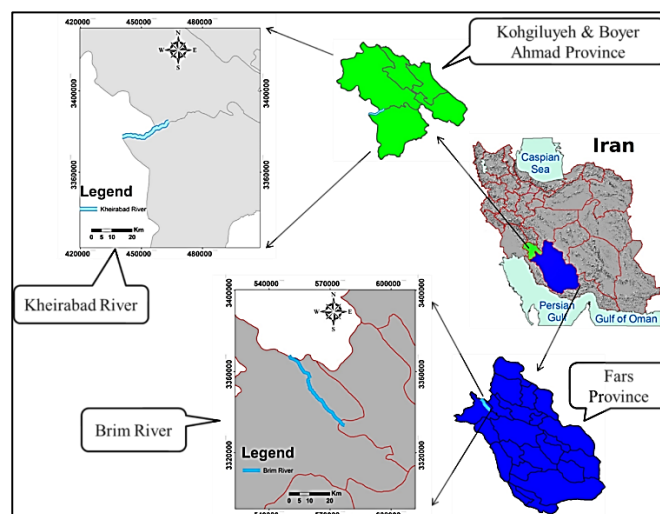
Annealing temperature (°C)	Primer sequence (5'→3')	Size range (bp)	Locus
55	F: ATAATCACAGCCCCGAGAG R: GGGTGGTGGAATATATTGGAAA	80-120	Bbar4
51	F: GAGCAACAGCTGCTGTAGGA R: GTCGGACCAACCTGAAAACCT	356-496	Bbar7
48	F: NED-AATACGAAACTACTTGGAATGGC R: GTGAAAAGGTCCAGTTAAAAGC	176-248	IC228
50	F: GGGTATAGGTGAAAAGGTCC R: ATACGAAACTACTTGGAATGGC	180-256	IC230
52	F: TCCACCATGACCATTTTTACATA R: GGTGTCTGGATCTCATCTTGAA	224-276	IC434
60	F: CGCAATGCATTCTCCAATCTCAA R: GACCCCACTCATCACTGCCTCTC	236-452	IC720

even distribution and co-dominant inheritance (Chistiakov et al. 2006; Chen et al. 2008; Sun et al. 2009). SSRs are useful markers to understand the genetic structure of populations, genetic diversity and history of the target species.

Inland waters have a high inherently potential to speciation and every day, we observe new report for presence of new loaches in Iranian inland waters and threats to their diversity (Freyhof & Serov 2000; Janko et al. 2003; Suzawa 2006; Abdoli et al. 2011; Esmaeili et al. 2014a,b,c; Freyhof et al. 2014). In aquatic ecosystems, loaches are benthic fish and directly effect on nutrient and energy cycles. It is necessary to distinguish the genetic structure populations of species in all of their geographical distributions in order to better formulate conservation programs, effective management and stock assessment of species. Hence, this study was carried out to investigate the genetic diversity of *Paraschistura* sp. populations collected from the two locations, Kheirabad and Brim Rivers (Zohreh River system) using microsatellite markers.

Materials and methods

Sample collection and DNA extraction: For the microsatellite analysis, a total of 60 samples (30 males and 30 females) of *Paraschistura* sp. were collected from two geographical locations, Kheirabad, Kohgiluyeh & Boyer Ahmad Province

**Fig.1.** Sampling localities of *Paraschistura* sp. in southwestern of Iran.

(30°31'N, 50°24'E) and Brim Rivers, Fars Province (31°19'N, 51°15'E) in July 2014 both in Zohreh River basin (Fig. 1). Phenol/chloroform extraction protocol (Hillis et al. 1996) was used to extract DNA from fin tissue of each sample.

PCR Amplification and electrophoresis: Six microsatellite loci, Bbar4, Bbar7 (Taylor et al. 2001), IC228, IC230, IC434 and IC720 (Bang et al. 2009) were used for the analysis of genetic diversity (Table 1). Amplification conditions for each locus were performed with a reaction volume of 25μL component according to Geng et al. (2006). The amplification cycle for all loci consisted of an initial denaturing at 94°C for 3min; 35 cycles of 94°C

Table 2. Polymorphic information content (PIC) across loci used in the present study

Locus	Bbar4	Bbar7	IC228	IC230	IC434	IC720
PIC	0.77	0.93	0.91	0.86	0.86	0.91

Table 3. Genetic characteristics of two populations of *Paraschistura* sp. based on 6 microsatellite loci in the studied locations*

		Bbar4	Bbar7	IC228	IC230	IC434	IC720
Kheirabad river	N _a	7	22	15	9	11	17
	N _e	5.212	12.447	10.687	6.151	5.977	14.297
	H _o	0.304	0.829	0.783	0.696	0.352	0.341
	H _e	0.813	0.922	0.906	0.837	0.833	0.930
	F _{IS}	0.622	0.196	0.137	0.169	0.583	0.673
	pHw	***	**	ns	***	***	***
Brim river	N _a	9	21	13	10	10	12
	N _e	3.712	14.901	9.121	6.151	6.260	7.723
	H _o	0.304	0.609	0.826	0.435	1.000	0.391
	H _e	0.731	0.933	0.890	0.837	0.840	0.871
	F _{IS}	0.583	0.348	0.072	0.481	-0.190	0.550
	pHw	***	**	***	ns	***	***

*N_a = number of alleles per locus; N_e = effective number of alleles; H_o = observed heterozygosity; H_e = expected heterozygosity; F_{IS} = inbreeding coefficient; pHW = Hardy-Weinberg probability test: *P<0.05; **P<0.01;

***P<0.001; ns = not significant were corrected with the sequential test of Bonferroni (Rice 1989).

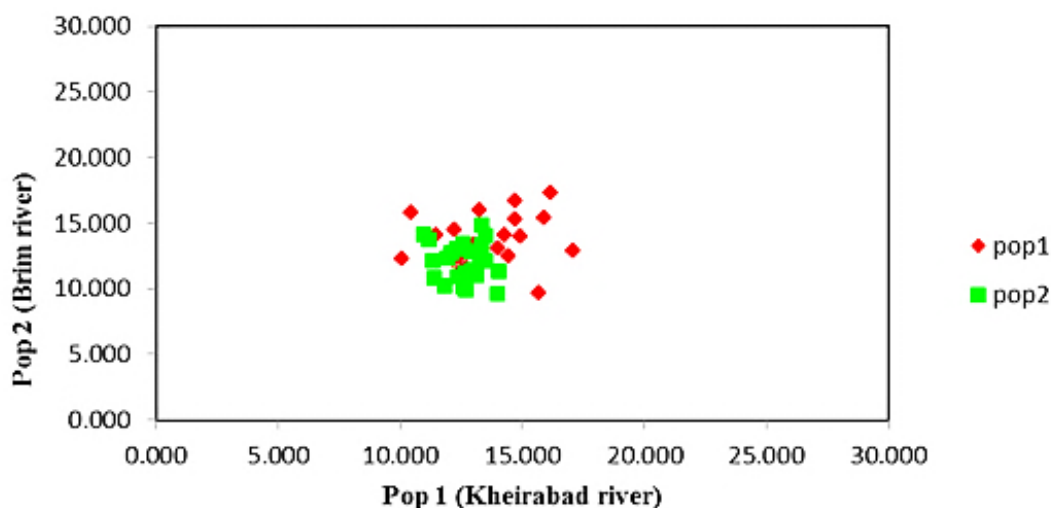
denaturing for 45s, 56°C annealing for 45s, 72°C extension for 2min; and a 5min final extension at 72°C. The PCR products were electrophoresed on 8 percent polyacrylamide gels stained with silver nitrate. (Rajora et al. 2000). As the molecular weight standard, a 100bp molecular weight marker (Fermentas) was used. The fragment lengths of the PCR products were determined using Gel-Pro Analyzer package (Ver 3.9, Gene, USA).

Data analysis: Genotyping errors due to null alleles and large allele dropout were checked using the Micro-checker software ver. 2.2.3 (Oosterhout et al. 2004). Deviation from Hardy-Weinberg equilibrium (HWE) was estimated by χ^2 test using Genepop software version 3.4 (Raymond & Rousset 2003) with exact P values being estimated using the Markov chain algorithm with 10,000 dememorization steps, 100 batches and 1,000 Iterations (Raymond & Rousset 2003). The significant levels in all cases with multiple tests were adjusted using the sequential Bonferroni correction (Rice 1989). The inbreeding coefficient (F_{IS}) and its

significance were calculated by FSTAT package ver 2.9.3 (Goudet 2001). The number of alleles (N_a), effective number of alleles (N_e), observed (H_o) and expected heterozygosities (H_e), Population Assignment Analysis and gene flow (Nm) between populations were computed using GenAlex 6.3 (Peakall & Smouse 2006). An analysis of molecular variance (AMOVA) based on F_{ST} index (Weir & Cockerham 1984) was used to examine the regional structure of genetic variation within and between two locations by GenAlex 6.3 (Peakall & Smouse 2006). Unbiased genetic similarity (I) and genetic distance (D) were calculated according to Nei (1978) by Popgene package ver 1.0 (Yeh et al. 1999). Statistical significance of an excess or defect of heterozygosity was estimated using the Mann-Whitney test. In testing the departure from mutation-drift equilibrium based on heterozygosity excess or deficiency for both populations, bottleneck analysis was conducted using Bottleneck Package version 1.2.02 under the TPM of microsatellites (Cornuet & Luikart 1996). Polymorphism Information Content (PIC) was

Table 4. Results of Analysis of Molecular Variance based on F_{ST} index.

Source	df	ss	MS	Est. Var.	%	Stat	value	P (rand >= data)
Among locations	1	6.38	6.38	0.08	3%			
Within locations	90	237.67	2.64	2.64	97%	Fst	0.027	0.010

**Fig.2.** Population Assignment analysis in *Paraschistura* sp. between Kheirabad and Brim rivers.

computed in the CERVUS software ver 3.0.3 (Botstein et al. 1980).

Results

All of the loci used in this research displayed a high level of polymorphism (Table 2). Based on the results obtained from Genepop software, it was distinguished that there were no signs of linkage disequilibrium between any pair of loci ($P > 0.05$). The average numbers of alleles in Kheirabad and Brim Rivers were 13.5 and 12.5, respectively. The lowest and highest observed allele numbers were seven and 22 alleles across Bbar4 and Bbar7 loci in Kheirabad River, respectively. Numbers of alleles and their distribution, effective number of alleles, inbreeding coefficient, as well as observed and expected heterozygosities are presented in Table 3. The average H_O between locations was obtained 0.524, whereas H_e was calculated 0.871 and this difference between H_O and H_e was statistically significant in Mann-Whitney test ($P < 0.05$). Observed heterozygosity value in Kheirabad river (0.53) was lower than expected heterozygosity value (0.87), as

well as Brim river ($H_O = 0.59$, $H_e = 0.85$). The results showed deviation from Hardy-Weinberg equilibrium at 10 out of 12 loci population tests (Table 3). Based on the results obtained of MICRO-CHECKER software did not detect scoring errors associated with large allele dropout in all six loci screened, but null alleles may exists (mean frequency estimate = 0.182). The mean value of N_m between two populations of Kheirabad and Brim was obtained 9.142 and the F_{ST} value was calculated 0.02. Lowest and highest values of N_m were observed in IC434 (6.103) and IC720 (17.009) loci, respectively. AMOVA based on F_{ST} index revealed that the variation between the two populations was only 3% of the total variance while the variation within populations was 97% (Table 4). Nei's genetic distance between the two populations was 0.629. No populations displayed significant heterozygosity excess in the sign test (P value for kheirabad = 0.55, P value for Brim = 0.25), standardized differences test (0.09 and 0.24, respectively) and Wilcoxon test (0.15 and 0.43, respectively). Finally, two studied populations in this research were approximately distinguished by

employing population assignment analysis (Fig. 2).

Discussion

All of the six loci used in this study showed highly polymorphism and there were no evidence of linkage disequilibrium between all pairs of loci ($P>0.05$) which indicated there were no any problem to use of these loci for *Paraschistura* sp. Heterozygosity is an evolutionary index in populations' dynamism and survival issues. The allelic richness is more useful tool than the heterozygosity, because the allele number is directly connected to effective population size (Nei et al. 1975; Diz & Presa 2009). In this research the average observed heterozygosity in Kheirabad river (0.53) was lower than value reported for freshwater fish ($H_o=0.54\pm0.25$; Dewoody & Avise 2000) but in Brim river this index was higher (0.59). In term of allelic richness, the mean numbers of alleles in Kheirabad and Brim rivers (13.5 and 12.5 alleles, respectively) are slightly higher than value reported for freshwater fish ($N_a=9.1\pm6.1$; Dewoody & Avise 2000). In small fish populations such as populations in closed inland waters and springs high allele frequency variance more occurs due to small effective population size. In other words, a natural fluctuation of allele frequency in small populations is much more, from one generation to another (Freeland et al. 2011).

The high observed deviation from Hardy-Weinberg equilibrium may be related to null alleles, high gene flow, genetic drift, inbreeding and small sample size (Bhassu et al. 2004; Borrell et al. 2008). Hardy-Weinberg equilibrium is based on random mating, so deviation from it is expected in small populations (Dixon et al. 2008). Many natural populations of fish have shown deviation from Hardy-Weinberg equilibrium (Castric et al. 2002; Yue et al. 2004; Lucentini et al. 2006; Bang et al. 2009). High migration and null alleles were reported as the main causes for high observed deviation in natural populations of *Alosa braschnikowi* in the Caspian Sea (Jafari et al. 2014). In the present study, inbreeding and null alleles likely are the main causes

for high observed deviation. There was significant deficiency in H_o rather than H_e at both Kheirabad and Brim populations. In natural fish populations deduction in H_o may happen due to non-random sampling, genetic drift, fishing pressure, human effects on water bodies or a combination of these factors (Skalla et al. 2004.; Li et al. 2007). In these rivers a combination of mentioned reasons may act. Inbreeding can affect the observed heterozygosity but do not have any effect on allelic diversity (Jafari et al. 2014). In this research, the mean value of inbreeding coefficient in Kheirabad River (0.41) was higher than Brim River (0.32) with corresponded to observed heterozygosity in these two locations. Based on the results from FSTAT software, FIS had a significant influence ($P=0.004$) on the deduction of H_o rather than H_e . In addition, when H_o is statistically lower than H_e , the Wahlund effect must be assumed. Deduction in H_o rather than H_e can be consider as a sign of genetic bottleneck presence in populations (Pan & Yang 2010). The results of genetic bottleneck analysis based on TPM model indicated that no populations displayed significant heterozygosity excess and deficiency ($P>0.05$), suggesting that both the Kheirabad and Brim populations have not experienced a recent bottleneck. This subject revealed the excellency of allelic richness in genetic population studies. The results obtained from this research were so close from the research applied on *Paraschistura* sp. from Shapour and Brim rivers (Askari & Shabani 2013).

Analysis of Molecular Variance (AMOVA) is a criterion for population structure examination, determining the level of genetic differentiation and genetic similarity between populations (Grassi et al. 2004). Way of propagation and nourish, life history, natural and artificial dams are some of the reasons which affect the genetic populations structure (Tiedemann et al. 2000). The F_{ST} index is a good measurement to determine population differentiation. The mean F_{ST} obtained value of 0.02 showed low levels of genetic differentiation between the two populations in concordance with the value

reported between populations of Shapour and Brim rivers by Askari & Shabani (2013). Based on the available data, high rate of gene flow was seen between two locations in this study ($N_m=9.14$), as well as between Brim and Shapour rivers ($N_m=11.34$, Askari & Shabani 2013). When $N_m>1$, the gene flow is the main component of genetic differentiation and if $N_m<1$, genetic drift is the main (Freeland et al. 2011). Genetic structure of *Paraschistura* sp. in these two rivers likely formed due to its historically gene flow and being part of a main River system (Zohreh). Adult fish usually spawn near their habitat and sediments and high velocity of water flow might damage the fish eggs. Low heterozygosity due to reducing the population size and increasing inbreeding coefficient is a characteristic key of these populations. Genetic distance obtained in this study ($D=0.629$) is a very important taxonomic tool. When genetic distance value among populations is ranged between 0.03 and 0.20, it indicates that the populations are of the same species (Thorp 1982). Thus, based on the results obtained in this study ($D=0.629$), it might be concluded that these two populations are probably two separate species but more phylogenetic studies such as using other molecular markers and osteology are needed to certainly decide on their taxonomy identity. Genetic studies on the other populations of *Paraschistura* in their entire distribution ranges are suggested for better and efficiency management fish diversity.

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